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Inhibition of Benzo(A)Pyrene Monooxygenase by alpha-Naphthoflavone may be Partially Mediated by the Metabolite 9-Hydroxy-alpha-Naphthoflavone

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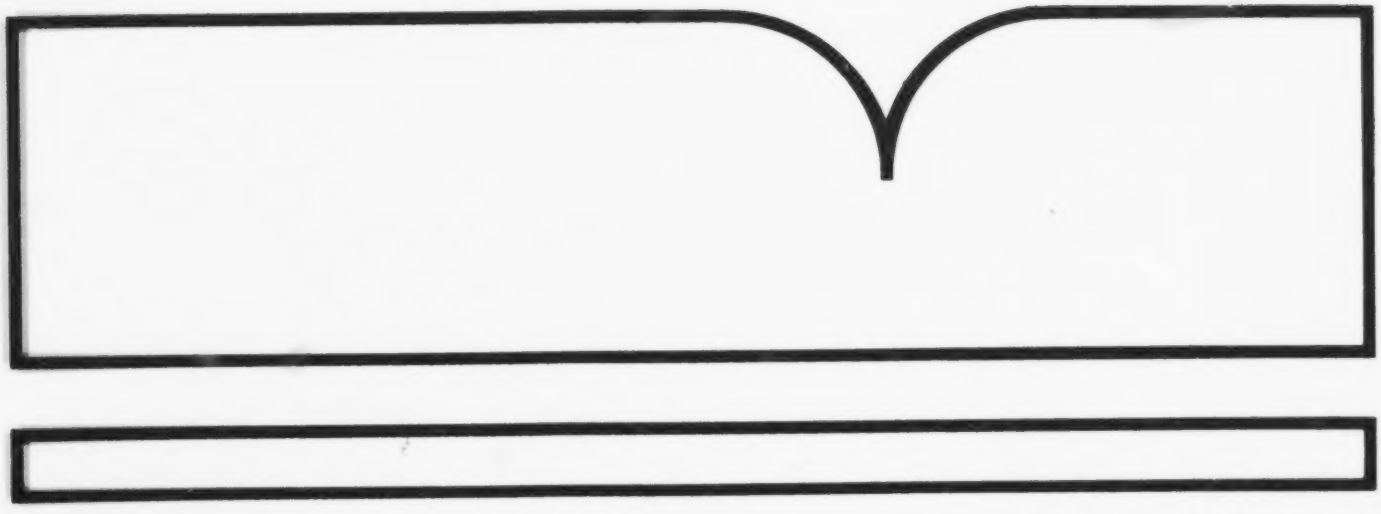

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16. ABSTRACT

β Naphthoflavone (ANF) inhibits β naphthoflavon-induced rat liver microsomal benzo(a)pyrene metabolism and is transformed by these microsomes into naphthoflavone metabolites. We determined the inhibitory effect on benzo(a)pyrene (B(a)P) metabolism of several of these metabolites and of 6-methoxy-ANF, using β naphthoflavone-induced rat liver microsomes. 9-Hydroxy-ANF was the most active inhibitor (I_{50} = 1.47 μM) and was metabolized from ANF by these hepatic cimrosomes in concentrations which are inhibitory. Therefore, 9-hydroxy-ANF a microsomal metabolite of ANF, may play a role in the inhibition of B(a)P oxidation by ANF.

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INHIBITION OF BENZO(A)PYRENE MONOOXYGENASE BY α-NAPHTHOFLAVONE MAY BE PARTIALLY MEDIATED BY THE METABOLITE 9-HYDROXY-α-NAPHTHOFLAVONE

(Mixed-function oxidases; metabolic activation; microsomes; 7,8-benzoflavone)

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SUMMARY

α-Naphthoflavone (ANF) inhibits β-naphthoflavone-induced rat liver microsomal benzo(a)pyrene metabolism and is transformed by these microsomes into α-naphthoflavone metabolites. We determined the inhibitory effect on benzo(a)pyrene (B(a)P) metabolism of several of these metabolites and of 6-methoxy-ANF, using β-naphthoflavone-induced rat liver microsomes. 9-Hydroxy-ANF was the most active inhibitor ($I_{50} = 1.47$ μM) and was metabolized from ANF by these hepatic microsomes in concentrations which are inhibitory. Therefore, 9-hydroxy-ANF a microsomal metabolite of ANF, may play a role in the inhibition of B(a)P oxidation by ANF.

INTRODUCTION

It has been previously demonstrated that α-naphthoflavone (7,8-benzoflavone), or ANF, can inhibit the metabolism of B(a)P and other substrates by hepatic microsomes from rats pretreated with β-naphthoflavone or 3-methylcholanthrene [1, 2].

ANF is metabolized by β-naphthoflavone-induced rat liver microsomes to ANF-5,6-oxide, 6-hydroxy-ANF, 7-hydroxy-ANF, 9-hydroxy-ANF, and 5,6-dihydro-5,6-dihydroxy-ANF [3, 4]. Coombs [5] has similarly reported the formation of

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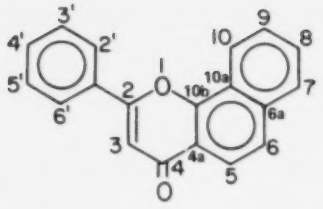
Abbreviations: ANF, α-naphthoflavone; B(a)P, benzo(a)pyrene.



ANF-5,6-oxide and has identified 9,10-dihydro-9,10-dihydroxy-ANF and 5-hydroxy-ANF in incubations of ANF with 3-methylcholanthrene-induced rat liver microsomes. The concept that metabolism of ANF might be required for its ability to inhibit β-naphthoflavone-induced rat liver microsomes has been proposed, based on a structure-activity analysis [2]. In that report, ANF derivatives with nonoxidizable substituents at position 6 were devoid of inhibitory activity, whereas those with oxidizable substituents were active. We report here the inhibitory activity of several synthetically prepared ANF metabolites and a related derivative on the metabolism of B(a)P and propose an explanation for the observed kinetic results of ANF reported by Wiebel and Gelboin [6] based on the metabolism of ANF to an active inhibitory species, 9-hydroxy-ANF.

MATERIALS AND METHODS

Chemicals and radiochemicals

[^{3}H]B(a)P and [^{3}H]ANF were purchased from Amersham (Arlington Heights, IL) and were purified by HPLC using a DuPont Instruments (Wilmington, DE) Model 848 chromatograph fitted with 4.6 mm i.d. × 25 cm Zorbax-ODS column. An isocratic solvent system of methanol was employed at a flow rate of 2.0 ml/min. Radiochemical purity was estimated to be >99% using this system. Authentic 6-hydroxy-ANF, 7-hydroxy-ANF, 9-hydroxy-ANF, and 6-methoxy-ANF were synthesized by modification of the method of Mahal and Venkataraman [7] using the appropriately substituted methoxy-2-acetyl-1-naphthols. The analytic and spectroscopic data of these synthetic phenols were fully consistent with their structure and will be reported elsewhere. ANF and spectrograde solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or from Burdick and Jackson (Muskegon, MI).

Preparation of microsomes

Male Charles River CD rats (45 days old) were maintained on lab chow and water ad lib. Groups of the least 4 rats were induced with β-naphthoflavone (Aldrich Chemical Co., Milwaukee, WI), 20 mg/kg in 0.25–0.5 ml corn oil, injected i.p. on

each of 4 successive days. Animals were starved for 24 h after the last treatment and microsomes prepared. They were stored according to Van der Hoeven et al. [8]. Protein concentrations were determined by the method of Lowry et al. [9], using bovine serum albumin as the protein standard (Sigma Chemical Co. St. Louis, MO).

Inhibition of B(a)P monooxygenase

B(a)P monooxygenase was assayed by the method of Nesnow [2]. Triplicate 1.0 ml incubation mixtures each contained: microsomal protein, 0.2 mg/ml; 1 μmol of NADP; 4.5 μmol of glucose-6-phosphate; 1.80 units of glucose-6-phosphate dehydrogenase; 3 μmol of $MgCl_2$; 50 μmol of potassium phosphate buffer, pH 7.50; and 60 nmol of [^{3}H]B(a)P specific activity 12–25 mCi/mmol, with and without inhibitor, as appropriate. Inhibitor was dissolved in 25 μl of acetone. Reaction was begun upon addition of substrate dissolved in 25 μl of acetone and, after 15 min of incubation at 37°C, was terminated by the addition of 1.0 ml of 0.5 N NaOH in 80% aqueous ethanol. Each sample was vortexed, 3.0 ml of spectrograde hexane was added, and the sample was vortexed again for 1.5 min. After centrifugation for 10 min at 2000 rev./min, an aliquot (0.3 ml) of the lower phase was removed, neutralized with 0.5 N HCl, Aquasol-2 added (New England Nuclear, Boston, MA), and the radioactivity was determined by liquid scintillation spectrometry. Each sample was corrected for background by subtracting the activity obtained using ethanolic NaOH-treated microsomes in the incubation mixture. Control incubations contained microsomes, NADPH generating system, buffer, [^{3}H]B(a)P, and 25 μl acetone. The concentration of inhibitor which causes a 50% reduction in enzymatic activity (I_{50}) was determined for each inhibitor by constructing a dose–response relationship between log concentration of inhibitor and enzyme activity as % of control. At least four concentrations of inhibitor were examined for each agent using concentration ranges of: 7-hydroxy-ANF, 1–50 μM; ANF, 6-hydroxy-ANF, 6-methoxy-ANF, 1–25 μM; 9-hydroxy-ANF, 0.5–3 μM.

Metabolism of ANF to 9-hydroxy-ANF

The metabolism of ANF was performed according to Nesnow and Bergman [3]. [^{3}H]ANF was incubated with microsomes and an NADPH-generating system according to the following procedure. A 5.0 ml incubation mixture contained 5 μmol NADP; 22.5 μmol glucose-6-phosphate; 9 units glucose-6-phosphate dehydrogenase; 15 μmol $MgCl_2$; 250 μmol potassium phosphate buffer (pH 7.50); 25 μM [^{3}H]ANF (spec. act., 1–2 μCi/μmol); and 2.00 mg microsomal protein. Reaction was begun at 37°C with agitation and the addition of substrate. The reaction was terminated at 15 min upon the addition of 15 ml of ethyl acetate–acetone (2:1). After thorough mixing and centrifugation at 800 × g, a 12 ml aliquot of the organic layer was removed and evaporated under dry nitrogen. The

samples were reconstituted in 75 μl of methanol and chromatographed on a 6.2 mm (i.d.) × 22.5 cm Zorbax-ODS column using a mixture of methanol–water (85:15) at a flow rate of 1.8 ml/min. Fractions (12 s) were collected directly into liquid scintillation vials for subsequent radiochemical analysis. 9-Hydroxy-ANF exhibited an elution volume of 9.5 ml.

RESULTS

Microsomes prepared from β-naphthoflavone-induced rats were incubated with [^{3}H]B(a)P and an NADPH generating system in the presence and absence of the naphthoflavones. The formation of B(a)P metabolites in this assay has been shown to be linear with time, B(a)P and protein concentrations under the conditions chosen [2]. This B(a)P monooxygenase assay measures up to 87% of all the B(a)P metabolites formed in the microsomal incubation [10]. All naphthoflavones examined (except for 7-hydroxy-ANF) exhibited similar dose–response inhibition curves, two of which are shown in Fig. 1. The three ANF metabolites 6-hydroxy-ANF, 7-hydroxy-ANF, and 9-hydroxy-ANF inhibited B(a)P monooxygenase to different extents (Table I). At concentrations up to 50 μM, 7-hydroxy-ANF did not inhibit greater than 50% of the control enzyme activity. Although 6-hydroxy-ANF and ANF inhibited B(a)P monooxygenase with I_{50} values which were statistically different ($P = 0.01$), these differences are probably not biologically significant. 6-Methoxy-ANF demonstrated inhibitory activity similar to that of its corresponding phenol ($P = 0.16$) and was not significantly different than ANF ($P = 0.4$). This result

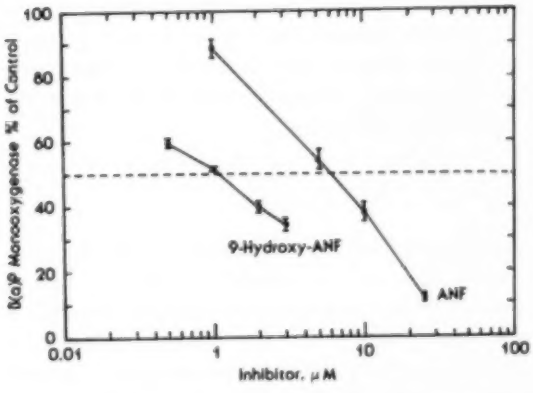


Fig. 1. Inhibition curves of B(a)P monooxygenase by ANF and 9-hydroxy-ANF. B(a)P monooxygenase assay was performed according to Nesnow [2] using liver microsomes prepared from β-naphthoflavone-induced rats. Triplicate determinations were performed at each inhibitor concentration using 60 μM [^{3}H]B(a)P, a protein concentration of 0.2 mg/ml, and a 15 min incubation. The specific activity of acetone-treated controls was 2–2.5 nmol [^{3}H]B(a)P oxidized/min/mg protein. Values are mean ± SD.

TABLE I

INHIBITION OF B(a)P MONOOXYGENASE BY α-NAPHTHOFLAVONES[a]

Naphthoflavone	I_{50}[b] (μM)
6-Hydroxy-ANF	4.54 ± 0.86
7-Hydroxy-ANF	>50
9-Hydroxy-ANF	1.47 ± 0.37
6-Methoxy-ANF	5.47 ± 1.25
ANF	5.98 ± 0.72

[a]See to legend Fig. 1 for details.

[b]I_{50} is the concentration of inhibitor which inhibits the enzymatic reaction by 50% and is expressed as mean ± SD for six replicate determinations. Statistical comparisons made by Students *t*-test.

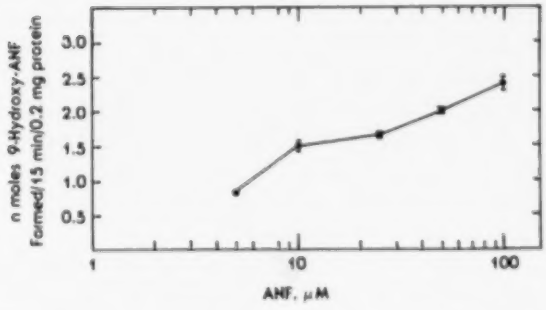


Fig. 2. The formation of 9-hydroxy-ANF from ANF by β-naphthoflavone-induced rat liver microsomes as a function of ANF concentration. Triplicate determinations were performed at each ANF concentration using [^{3}H]ANF, 0.4 mg/ml microsomal protein, and a 15 min incubation. Values are mean ± SD.

indicates an additional example of a naphthoflavone with an oxidizable substituent at position 6 which retains its inhibitory activity [2]. 9-Hydroxy-ANF was the most potent inhibitor with an I_{50} of 1.47 μM, significantly less than ANF ($P<0.001$).

The metabolism of ANF to 9-hydroxy-ANF by β-naphthoflavone-induced rat liver microsomes was carried out under incubation conditions (without B(a)P) similar to those in the monooxygenase assay with respect to cofactor and buffer concentrations and incubation time. Protein concentrations were increased twofold for increased product formation, since previous experiments had shown no change in specific activity of ANF metabolite formation between 0.2 and 0.4 mg/ml microsomal protein. 9-Hydroxy-ANF formation was directly related to ANF concentration from 5–100 μM ANF (Fig. 2). When these experiments were carried out in the presence of 60 μM B(a)P, there was no significant change in the formation of 9-hydroxy-ANF.

DISCUSSION

The relationship between the metabolism of ANF and the ability of ANF to inhibit hepatic microsomal mixed-function oxidases was examined by measuring the inhibitory properties of several ANF metabolites. Based on the I_{50} values, 9-hydroxy-ANF proved to be the most potent inhibitor of B(a)P metabolism of all flavones studied and was four times more active than ANF.

Wiebel and Gelboin's [6] kinetic data on the inhibition of B(a)P metabolism by ANF indicate that at 3.3. and 10 μM ANF there are significant deviations from linearity in the Lineweaver–Burk plots; this suggests an inhibiting mechanism that is more complex than competitive inhibition. Our data on 9-hydroxy-ANF formation indicate that at 5 and 10 μM ANF there are 0.8 and 1.5 μM 9-hydroxy-ANF formed, respectively. These concentrations of 9-hydroxy-ANF are capable of inhibiting 45–55% of B(a)P monooxygenase activity and should have an effect on the kinetic pattern of inhibition shown by ANF. In addition, there is a close relationship between: (1) the extent of inhibition of B(a)P monooxygenase at a given concentration of ANF (Fig. 1), (2) the concentration of 9-hydroxy-ANF formed at that given concentration of ANF (Fig. 2), and (3) the extent of inhibition of B(a)P monooxygenase at that concentration of 9-hydroxy-ANF. These findings suggest that a major part of the inhibitory activity of ANF can be ascribed to the concentration of 9-hydroxy-ANF formed by the microsomes.

All of these factors suggest an active role of 9-hydroxy-ANF, a microsomal metabolite of ANF, in the mechanism of inhibitory action of ANF in β-naphthoflavone-induced rat liver microsomes.

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